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High Glucose Concentration in Isotonic Media Alters Caco-2 Cell Permeability

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ABSTRACT

Caco-2 cell permeability was evaluated in isotonic media containing high (25mM) or physiological (5.5mM) glucose concentrations. Transepithelial electrical resistance (TEER) and membrane fluidity were measured to assess glucose-induced alterations in physical barrier properties. In parallel, distribution of the actin filament (F-actin) and zonula occludens-1 (ZO-1) proteins was assessed by confocal microscopy. Transepithelial fluxes of mannitol, hydrocortisone, digoxin, and glycyl sarcosine (Gly-Sar) that permeate the intestinal mucosa by various pathways were measured to quantify the effect of glucose-induced changes on Caco-2 cell permeability. High glucose decreased maximum TEER of cell monolayers by 47%, whereas membrane fluidity at the hydrophobic core and lipid/polar head interphase was significantly increased. F-actin distribution in high glucose cells appeared more diffuse while ZO-1 was unchanged. Mannitol and hydrocortisone fluxes across Caco-2 cells cultured in high glucose increased by 65% and 24%, respectively. In addition, high glucose decreased the maximum transport capacity (Vmax) of PepT-1. P-glycoprotein activity, however, was unchanged. In conclusion, high extracellular glucose concentration in isotonic media significantly alters physical barrier properties of Caco-2 cell monolayers, which predominantly affects transepithelial transport of solutes permeating the cell barrier by paracellular and transcellular passive diffusion and facilitated transport mediated by the proton-dependent oligopeptide transporter (PepT-1).

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INTRODUCTION

Modern screening strategies employed in drug development integrate information from various in vitro analyses that focus on physicochemical properties, membrane permeation properties as well as chemical and enzymatic stability of new chemical entities (NCEs). This approach results in successful identification of lead compounds with desired pharmacological and biopharmaceutical/pharmacokinetic properties and facilitates prediction of potential formulation difficulties.[1] Recent advances in combinatorial chemistry, computational modeling, genomics, and proteomics have dramatically increased the number of NCEs and, therefore, require further streamlining of the screening process. As an alternative, it was proposed to include in the selection of lead compounds a significant computational component that is based on quantitative structure-transport relationships.[2-4] However, successful implementation of this in silico approach critically depends on reliable databases composed of physicochemical properties, membrane permeation properties, and information on stability of a diverse array of chemical entities.

Over the past decade, the Caco-2 cell culture model has been validated as a suitable in vitro system to assess intestinal permeation properties of NCEs that are predictive for in vivo absorption.[5-7] Therefore, the Caco-2 cell culture model has been adopted as a major tool in many preclinical screening programs to obtain quantitative experimental information on membrane permeation properties of solutes, which presumably can be used in the development of computational models for in silico screening of NCEs.[5,8] However, it is well documented

that experimental factors such as filter support, culture conditions, passage number and serum supplements result in morphological alterations of Caco-2 cell monolayers.[9-12] As a consequence, apparent permeability coefficients (P_{app}) determined for the same solute in different laboratories vary considerably, which reduces the predictive power of computational methods based on these data. Routinely, Caco-2 cells are cultured in the presence of high glucose (25mM) to promote rapid growth and differentiation.[9-13] High extracellular glucose, however, has been demonstrated to induce significant changes in cellular processes.[14,15] Recently, our laboratory reported that the maximum transport capacity of the oligopeptide transporter PepT-1, which is expressed in the apical membrane of Caco-2 cells, significantly decreased when cells were exposed to 25mM extracellular glucose for at least 2 hours.[16,17] Further studies revealed that the underlying mechanism of altered functional activity of this carrier involves, at least in part, an oxidative pathway. In addition, activation of the protein kinase C (PKC) signaling pathway may also be induced by high extracellular glucose in Caco-2 cells.[16] However, oxidative stress has been shown to disrupt the cytoskeleton and alter membrane fluidity of this in vitro model of the intestinal mucosa, which significantly affects permeation of solutes.[18-21]

The objective of the present study was to evaluate the effect of nonphysiological glucose concentration in isotonic media on barrier properties of Caco-2 cell monolayers restricting paracellular and transcellular solute transport. Glucose-induced alterations at the tight junction area were assessed using immunofluorescent analysis of actin filament (F-actin) and zonula occludens-1 (ZO-1) distribution as well as transepithelial electrical resistance (TEER) measurements. Membrane fluidity at the hydrophobic core and the lipid/polar head interphase was determined by fluorescence polarization. Finally, transepithelial transport of mannitol, hydrocortisone, digoxin, and glycyl sarcosine (Gly-Sar) were determined to quantify the effects of glucose-induced changes in physical barrier properties of Caco-2 cell monolayers on the flux of solutes permeating the intestinal mucosa by various pathways (ie, paracellular and transcellular passive diffusion, transcellular transport with substrate activity for apical efflux systems such as P-glycoprotein, and transcellular carrier-mediated transport).

MATERIALS AND METHODS

Materials

[^{14}C]-D-Mannitol (53 mCi/mmol) and [^{3}H]-Gly-Sar (4 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). [^{3}H]-hydrocortisone (50 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO). [^{3}H]-digoxin (37 Ci/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA). The dipeptide Gly-Sar was obtained from Bachem Bioscience (Bubendorf, Switzerland). Hanks' Balanced Salts were purchased from Sigma (St Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM) (50-003-PB, 25mM glucose, 335 ± 30 mOsm/kg and 50-014-PB, 5.5mM glucose, 335 ± 30 mOsm/kg), L-glutamine 200mM (100X), penicillin (10 000 IU/mL), streptomycin (10 000 µg/mL), and nonessential amino acids 10mM (100X) in 0.85% saline were purchased from Mediatech (Herndon, VA). Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA). Bio-Rad dye reagent concentrate was obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals were of high purity or analytical grade and used as received.

Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 18. The cells were routinely maintained in DMEM containing either 25mM (high) or 5.5mM (physiological) glucose and supplemented with 1% L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1% nonessential amino acids and 10% heat-inactivated fetal bovine serum at 37°C in a controlled atmosphere of 5% CO_2 and 90% relative humidity. Experiments were performed using Caco-2 cells between passages 60 and 78, which were adapted for at least 5 passages to the different glucose concentrations. For transport studies, 2.5×10^5 cells/well were seeded on collagen-coated polycarbonate membranes (Transwell 24.5 mm in diameter, 3 µm pore size) (Costar Corp, Cambridge, MA).

Transepithelial Electrical Resistance

Effect of high and physiological glucose conditions on TEER of Caco-2 cell monolayers was measured in triplicate over a period of 4 weeks using an EVOM epithelial voltohmmeter equipped with an Endohm electrode chamber (World Precision Instruments, Sarasota, FL). TEER of the collagen-coated filter membrane without cells was subtracted from experimental readings before correcting for the surface area of the filter (4.71 cm^2).

Fluorescence Microscopy

Caco-2 cell monolayers cultured on Transwells (Corning Costar, Cambridge, MA) for at least 21 days in the presence of 25mM and 5.5mM glucose were washed 3 times with phosphate buffered saline (PBS), pH 7.4, and fixed for 20 minutes at room temperature using freshly prepared 3.75% paraformaldehyde solution. Cells were washed with PBS and then permeabilized for 30 minutes at room temperature using 0.2% Triton-X-100 in PBS. Cells were washed with PBS and incubated for 3 hours at room temperature with rabbit anti-ZO-1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:50 in 5% normal goat serum in PBS (NGS/PBS). After 3 washes with 5% NGS/PBS, the cells were co-incubated for 1 hour at room temperature with secondary antibody (Alexa Fluor 488 goat anti-rabbit, 1:400) and Alexa Fluor Phalloidin 546 (1:200) (Molecular Probes, Eugene, OR) prepared in 5% NGS/PBS. At the end of this incubation, the cells were washed 2 times with 5% NGS/PBS and once with PBS, and mounted using Gel Mount media (Fisher Scientific, Pittsburgh, PA). Slides were visualized by confocal laser scanning microscopy using a Zeiss LSM510 equipped with argon and helium neon (HeNe) lasers, and a Plan-Apochromat 63×/1.4 Oil DIC objective (Carl Zeiss MicroImaging, Thornwood, NY). Alexa Fluor Phalloidin 546 was visualized by excitation with the HeNe laser and at emission wavelengths between 590 and 620 nm, whereas Alexa Fluor 488 was visualized by excitation with the argon laser and at emissions between 500 and 540 nm. All images were captured under identical microscope settings and processed using the LSM 510 software.

Membrane Fluidity Measurement

Confluent Caco-2 cell monolayers cultured for at least 21 days under high and physiological glucose conditions in 25 cm^2 tissue culture flasks were washed 3 times with PBS, trypsinized, and resuspended in PBS at a density of 2×10^5 cells/mL. Fluorescence anisotropy was determined as described previously[22] using 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethyl ammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluen esulfonate (TMA-DPH). Briefly, 2.5 mL of the Caco-2 cell suspensions were labeled in the dark at room temperature using either 2.5 µL of 1mM DPH in tetrahydrofuran for 30 minutes or 1mM TMA-DPH in dimethyl formamide for 2 minutes. Fluorescence polarization of the probes was determined with filters in the parallel and perpendicular orientations using a Hitachi 3500 spectrofluorometer with excitation and emission wavelengths set at 360 nm and 430 nm, respectively. The perpendicular component of fluorescence intensity was corrected for the intrinsic light polarization of the fluorometer, and fluorescence anisotropy (r) was calculated using the following equation,

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities measured in the directions parallel and perpendicular to the polarized exciting light, respectively. Cholesterol (5-50µM) and benzyl alcohol (5-30mM) were used as positive controls for these experiments. Benzyl alcohol ≥20mM increased membrane fluidity by decreasing DPH and TMA-DPH anisotropy by at least 6% and 2%, respectively. In contrast, cholesterol ≥20µM decreased membrane fluidity by increasing DPH and TMA-DPH anisotropy by at least 2% and 5%, respectively.

Transepithelial Transport

Caco-2 cells cultured on collagen-coated polycarbonate filters (Transwells) in the presence of high and physiological glucose concentrations were used between 21 and 28 days postseeding as described previously.[23] Prior to the experiment, cell monolayers were washed with Hanks' Balanced Salt Solution (HBSS, pH 7.4). Transport experiments with mannitol, hydrocortisone, and digoxin were initiated by adding the radioactive solute dissolved in HBSS, pH 7.4, to the donor compartment and HBSS, pH 7.4 to the receiver compartment (apical [AP] = 1.5 mL, basolateral [BL] = 2.6 mL). Aliquots were removed from the receiver (120 µL) and donor (20 µL) compartments at regular time intervals up to 120 minutes. Radioactivity in these samples was determined by liquid scintillation counting using the Beckman LS-6500 (Beckman Instruments, Fullerton, CA). The volume from the receiver compartment was always replaced with fresh, prewarmed HBSS, pH 7.4. Apparent permeability coefficients (P_{app}) of the solutes were calculated according to Equation 2.

$$P_{app} = \frac{\Delta Q/\Delta t}{C_0 \times A} \quad (2)$$

where $\Delta Q/\Delta t$ indicates linear appearance rate of mass in the receiver compartment; C_0, initial solute concentration in the donor compartment; and A, surface area (ie, 4.71 cm^2).

Transepithelial transport kinetics of Gly-Sar was measured using the above described protocol with the following modifications. Cell monolayers were preincubated at 37°C in the presence of Earle's Balanced Salt Solution (EBSS, pH 6.0) in the AP compartment and HBSS, pH 7.4, in the BL compartment. After 15 minutes, transport experiments were initiated by replacing the AP solution with 1.5 mL Gly-Sar (0.01mM-10mM) prepared in EBSS, pH 6.0, that contained a trace amount of [^{3}H]-Gly-Sar. Transport was terminated after 90 minutes by the addition of ice-cold HBSS, pH 7.4. Cell monolayers were dissolved in 1N NaOH and intracellular [^{3}H]-Gly-Sar amounts were quantified as described above. Total protein content was determined using the commercial Bradford protein assay.

Apparent substrate binding affinity (Km), maximum transport capacity (Vmax), and apparent diffusion coefficient (Kd) for this PepT-1 substrate were determined by nonlinear regression analysis using the following equation (Prism 3.0, Graph Pad Software, San Diego, CA):

$$V = \frac{Vmax \times [S]}{Km + [S]} + Kd \times [S] \quad (3)$$

where V indicates velocity of carrier-mediated transport, Vmax, maximum transport capacity; [S], concentration of Gly-Sar; Km, substrate binding affinity (Michaelis-Menten constant); and Kd, apparent diffusion coefficient.

Statistical Analysis

All experiments were carried out in triplicate and were repeated at least twice using different cell batches. Results are reported as mean ± SD. Significant statistical differences between two groups were evaluated using the unpaired Student t test ($P < .05$).

RESULTS

Effect of Extracellular Glucose on the Paracellular Barrier

TEER values of Caco-2 monolayers cultured in the presence of 25mM or 5.5mM glucose increased proportionally with days in culture (**Figure 1**). Significant differences in ion flux between the 2 culture conditions were observed after 12 days. The maximum TEER measured for these cell monolayers between 24 and 28 days are reported in **Table 1**. Cells cultured in isotonic media containing physiological glucose concentration exhibited a 2-fold greater resistance than monolayers maintained in isotonic media that was supplemented with 25mM glucose. This implies that barrier properties restricting paracellular diffusion are less developed in high glucose cells. However, the extracellular glucose concentration did not dramatically change the time required to reach the maximum TEER plateau (~24 to 28 days).

To correlate glucose-induced changes in TEER with morphological characteristics of the cell monolayers, distribution of the cytoskeletal element F-actin and tight junction protein ZO-1 were examined by confocal laser scanning microscopy (**Figure 2**). When cultured in 5.5mM glucose, the actin cytoskeleton shows a continuous ring appearance between adjacent cells, whereas in high glucose the actin staining appears discontinuous and less ordered (**Figure 2A**). Staining for the tight junction protein ZO-1, in contrast, revealed a diffusely punctuated protein distribution at the cell borders (**Figure 2B**), which was not affected by different extracellular glucose concentrations. Furthermore, it is important to note that the shape of Caco-2 cells cultured in the presence of 25mM and 5.5mM glucose appears similar. These morphological indications are consistent with reduced TEER values of cell monolayers maintained in 25mM glucose and suggest that high glucose reduces the physical barrier properties of the paracellular pathway in Caco-2 cell monolayers.

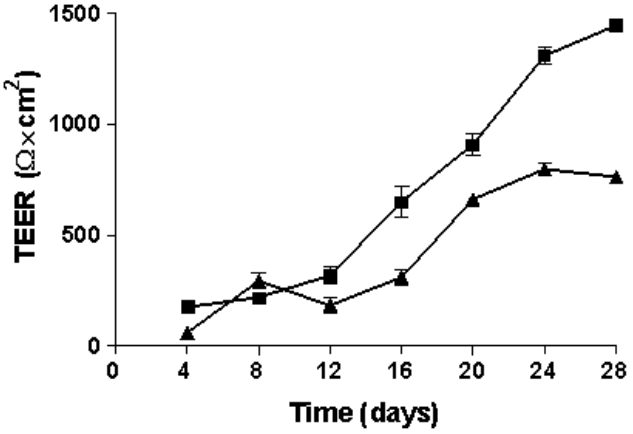

Figure 1. Effect of extracellular glucose concentration on TEER of Caco-2 cell monolayers. Transepithelial electrical resistance was measured over a 4-week period using Caco-2 cell monolayers that were maintained in isotonic media containing high (▲) or physiological (■) concentrations of glucose. Values are represented as mean ± SD (n = 3).

Table 1. Physical Barrier Properties of Caco-2 Cells Cultured Under High and Physiological Glucose Conditions*

Barrier		25mM Glucose†	5.5mM Glucose†	5.5mM Glucose + 20mM Mannitol†
Paracellular	$TEER_{max}$ [$\Omega \times cm^2$]	765 ± 20‡	1449 ± 14	ND
Transcellular	Anisotropy§			
	DPH	0.255 ± 0.001‡	0.263 ± 0.0001	0.262 ± 0.001
	TMA-DPH	0.339 ± 0.001‡	0.355 ± 0.002	0.357 ± 0.002

*DPH indicates 1,6-diphenyl-1,3,5-hexatriene; ND, not determined; TEER, transepithelial electrical resistance; and TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate.
† Experiments were determined in triplicate and results are reported as mean ± SD.
‡ Significantly different from the corresponding physiological glucose (5.5mM) values ($P < .05$).
§ Expressed as anisotropy units.

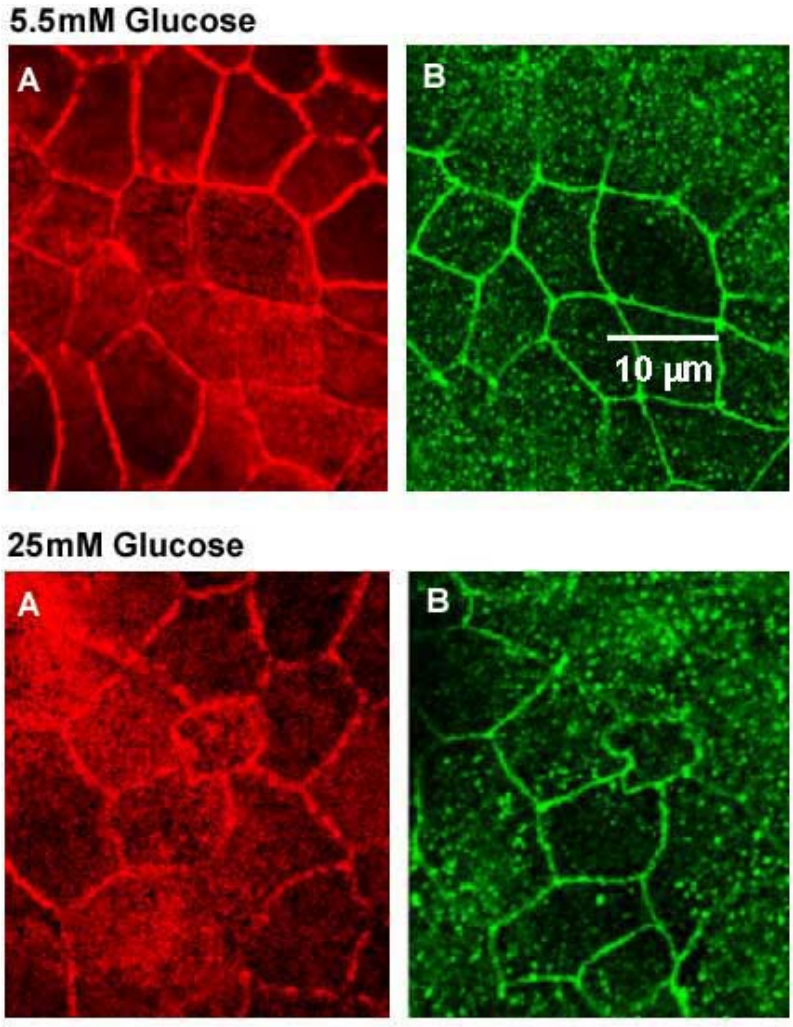

Figure 2. Confocal microscopy of tight junction complexes of Caco-2 cell monolayers. Differentiated Caco-2 cells cultured on Transwells for 25 days in the presence of 25mM or 5.5mM glucose were fixed and permeabilized as described in Materials and Methods. Tight junctions were visualized by (A) actin staining using Alexa Fluor Phalloidin 546 and (B) staining of ZO-1 proteins using a rabbit anti-ZO-1 monoclonal antibody followed by Alexa-Fluor 488 goat anti-rabbit antibody.

Table 2. Effect of Extracellular Glucose on Permeation of Marker Solutes across Caco-2 Cell Monolayers*

Marker	P_{app} [cm/sec] × 10^{-6}		
	25mM Glucose†	5.5mM Glucose†	5.5mM Glucose + 20mM Mannitol†
Mannitol	0.71 ± 0.01‡	0.43 ± 0.05	0.39 ± 0.03
Hydrocortisone	25.2 ± 0.25‡	20.4 ± 0.64	20.8 ± 1.04
Digoxin (AP→BL)	1.27 ± 0.05‡	0.99 ± 0.05	ND
Digoxin (BL→AP)	11.9 ± 0.45‡	9.66 ± 0.03	ND

* AP indicates apical; BL, basolateral; and ND, not determined.
† Experiments were determined in triplicate and results are reported as mean ± SD.
‡ Significantly different from the corresponding physiological glucose (5.5mM) values ($P < .05$).

Effect of Extracellular Glucose on the Transcellular Barrier

Transepithelial transport of solutes via the transcellular route is predominantly restricted by the barrier properties exhibited by the cell membrane. Membrane fluidity of the Caco-2 cell monolayers maintained in the presence of 25mM, 5.5mM glucose, or 5.5mM glucose plus 20mM mannitol was assessed using 2 different fluorescent probes that localize into different regions of the bilayer. High glucose significantly decreased the fluorescence anisotropy of TMA-DPH-labeled Caco-2 cell membranes (**Table 1**). Since anisotropy is inversely related to membrane fluidity, this result suggests that high extracellular glucose increases the mobility of the phospholipid groups at the lipid/polar head interphase. Similarly, a highly significant decrease in anisotropy was obtained for the hydrophobic core of the bilayer using DPH as probe (**Table 1**). In contrast, the membrane fluidity of cell monolayers maintained in 5.5mM glucose plus 20mM mannitol did not differ from cells maintained in physiological glucose media. As a consequence, we conclude that high glucose and not a structurally related compound such as mannitol, decreases physical barrier properties restricting transcellular permeability.

Extracellular Glucose and Passive Diffusion

In general, transepithelial flux across Caco-2 cell monolayers results from various permeation pathways accessible to the solute of interest.[24] To delineate the effect of different extracellular glucose concentrations on major permeation pathways, marker solutes were selected that are widely accepted to traverse the intestinal mucosa predominantly via one specific permeation pathway. The hydrophilic, uncharged mannitol (molecular weight [MW] 182.2) was used a marker for paracellular passive diffusion, whereas the more lipophilic, uncharged hydrocortisone (MW 362.5) was selected as a marker for transcellular passive diffusion. High glucose concentration significantly increased the P_{app} of both solutes across Caco-2 cell monolayers (**Table 2**). These results are consistent with our assessment of physical barrier properties restricting permeation of solutes via these 2 pathways. Since the presence of 5.5mM glucose + 20mM mannitol did not alter passive diffusion of the paracellular marker mannitol and the transcellular marker hydrocortisone (**Table 2**), we concluded that the changes in solute flux were the result of increased levels of glucose alone.

Extracellular Glucose and Transporter Activity

The effect of high extracellular glucose on the functional activity of integral membrane proteins facilitating transcellular transport of solutes was evaluated using digoxin, which exhibits substrate activity for the efflux system P-glycoprotein (P-gp), and Gly-Sar, which is a substrate for the intestinal oligopeptide transporter PepT-1. Bidirectional transport of digoxin in Caco-2 cell monolayers cultured in the presence of 5.5mM or 25mM glucose revealed a significant increase in the P_{app} values for both directions (**Table 2**). However, the net secretory permeability (ie, $P_{app\ BL\rightarrow AP}$ /$P_{app\ AP\rightarrow BL}$) calculated for this P-gp substrate in the presence of 25mM glucose was 9.4. Using cells cultured in the presence of 5.5mM glucose, this ratio was 9.8 suggesting that the functional activity of this membrane efflux system was not dramatically affected by these different glucose conditions.

The functional activity of the PepT-1 was assessed by determining the maximum transport capacity and bind-

Table 3. Effect of Extracellular Glucose Concentrations on Transepithelial Transport Kinetics of Gly-Sar across Caco-2 Cell Monolayers*

	25mM Glucose†	5.5mM Glucose†
Vmax [nmol/mg prot/min]	16.67 ± 7.19‡	31.95 ± 5.63
Km [mM]	0.45 ± 0.33	1.23 ± 0.35
Kd [nmol/mg prot/min/mM]	11.58 ± 0.83‡	8.82 ± 0.49

* Gly-Sar indicates glycly sarcosine; Kd, apparent diffusion coefficient; Km, substrate binding affinity; and Vmax, maximum transport capacity.

† Experiments were determined at least in triplicate and results are reported as mean ± SD.

‡ Significantly different from the corresponding physiological glucose (5.5 mM) values ($P < .05$).

ing affinity for the metabolically stable substrate, Gly-Sar. Carrier-mediated contribution to the overall flux of Gly-Sar was delineated by nonlinear regression analysis using Equation 3. The data shown in **Table 3** illustrate that increased concentration of extracellular glucose decreased the maximum transport capacity (Vmax) of this carrier by 48% without altering substrate affinity (Km) of this PepT-1 substrate. In addition, the passive component of transepithelial Gly-Sar flux across the cell monolayer as represented by Kd significantly increased when Caco-2 cells were cultured in the presence of 25mM glucose. These results are in agreement with earlier kinetic studies performed by our laboratory that focused on the carrier-mediated uptake of Gly-Sar across the apical membrane of Caco-2 cells after short-term and long-term exposure to high glucose.[16,17] The different effects of high glucose on functional activity of P-gp and PepT-1 imply protein-specific susceptibility of intestinal transporters to extracellular glucose.

DISCUSSION

The success of an in silico approach to predict intestinal permeability of NCEs critically depends on validated input data used to correlate chemical structure with membrane transport properties. Since the ultimate goal of computational preclinical screening is to select viable drug candidates for clinical studies in a time- and resource-efficient manner, the most preferred parameter describing intestinal permeability should have a significant correlation to the fraction absorbed from the intestinal tract in humans.

Absorption across the intestinal mucosa is influenced by a multitude of transport mechanisms available to the solute.[24] The use of in vitro cell culture systems such as the increasingly popular Caco-2 cell line has made it feasible to better understand this multivariate process.[25,26] Under standard culture conditions, Caco-2 cells spontaneously differentiate to exhibit morphological and biochemical features similar to the intestinal mucosa in vivo.[13] Therefore, this cell culture model is widely used in preclinical screening as a predictive tool to estimate oral bioavailability of NCEs.[5-7,27] However, recent correlation analyses suggest that P_{app} values $< 5 \times 10^{-6}$ cm/sec obtained across Caco-2 cell monolayers poorly estimate gastrointestinal absorption in humans.[28] Therefore, it is conceivable that these limitations will restrict future *in silico* approaches to successfully select viable candidates for drug development.

Recently, our laboratory reported that short- and long-term exposure of Caco-2 cells to elevated extracellular glucose significantly decreased the functional activity of the oligopeptide carrier (PepT-1). This decrease in activity was mediated, at least in part, via an oxidative pathway.[16,17] In this study, TEER values measured across cell monolayers that were maintained in the presence of 25mM glucose were decreased by 47% as compared with control cell monolayers cultured in physiological glucose. This was paralleled by a 67% increase in mannitol flux suggesting glucose-induced alterations at the tight junctions, which control flux via the paracellular pathway.[29] Morphological evaluation of Caco-2 cell monolayers using confocal microscopy revealed partial disruption of the F-actin ring in cell monolayers exposed to 25mM glucose, whereas distribution of the tight junction protein ZO-1 was not changed. Increased paracellular flux is generally the result of changes in the area represented by the paracellular space. In contrast to the hypothesis proposed by Pappenheimer and coworkers who reported that a high luminal glucose stimulus increased fluid absorption and decreased resistance at the tight junctions,[29-31] glucose-induced alterations in paracellular barrier properties of Caco-2 cell monolayers described in this study are most likely not the result of osmosis since both culture media containing either 5.5mM and 25mM glucose were iso-osmotic. Our laboratory demonstrated

that iso-osmotic culture media containing 25mM glucose induces significant production of reactive oxygen intermediates (ROI).[16] This finding supports our hypothesis that disruption of the cytoskeleton and changes in the tight junction area are secondary effects of glucose-induced formation of ROI. Earlier, hydrogen peroxide and other ROI have been shown to alter paracellular barrier properties and increase paracellular flux.[18,19,21] Nevertheless, direct interaction of ROI with ZO-1 or other tight junction proteins cannot be excluded. Future studies will be designed to determine the quantitative relationship between glucose-induced alterations at the paracellular junction and transepithelial fluxes of paracellular solutes exhibiting different molecular sizes.

High glucose significantly increased membrane fluidity at the lipid/polar head interface and the hydrophobic core of the bilayer. Increased fluidity implies a less-hindered conduit for lipophilic solutes to move across the membrane barrier, which was experimentally confirmed using hydrocortisone as a marker. Nevertheless, to clearly define the impact of glucose-induced changes in membrane fluidity on passive transcellular diffusion, additional transport experiments using a homologous series of solutes with different physicochemical properties (eg, molecular size, lipophilicity) are required. Earlier, Podolin and coworkers investigated the effect of a high sucrose diet on the lipid fluidity of liver sinusoidal membranes.[32] Long-term exposure of this disaccharide decreased the membrane fluidity suggesting a negative impact on membrane permeation. Although differences in lipid composition between sinusoidal and intestinal membranes may explain this apparent contradiction, it is more likely that chemical differences between glucose and sucrose are the sources of different mechanisms leading to alterations in membrane fluidity. As an example, Jourd'heuil and colleagues demonstrated that changes in the hemileaflet fluidity of brush border membranes induced by oxidative stress decreased the activity of the Na^+-dependent glucose transporter.[20]

We determined the impact of different extracellular glucose concentrations in iso-osmotic media on the functional activity of P-gp using digoxin as a model substrate. Digoxin has been shown to permeate Caco-2 cell monolayers predominantly via passive transcellular diffusion modified by substrate activity for P-gp.[33] Although transepithelial flux of this P-gp substrate was significantly increased across Caco-2 cell monolayers maintained in 25mM glucose, the calculated secretory permeability was not different from control cells cultured in physiological glucose. This implies that the increase in bidirectional digoxin flux was rather the result of glucose-induced alterations in barrier properties restricting passive transcellular diffusion than a direct or indirect effect on the membrane efflux protein.

In contrast, high extracellular glucose dramatically decreased Vmax of PepT-1 for Gly-Sar without affecting substrate binding affinity (Km). This is consistent with earlier observations from this laboratory that identified a glucose-induced decrease in cellular uptake kinetics of Gly-Sar mediated via an oxidative pathway.[16,17] The significantly increased apparent diffusion coefficient (Kd) estimated for passive transepithelial transport of Gly-Sar across Caco-2 cell monolayers appears to be the result of glucose-induced alterations in barrier properties restricting paracellular and transcellular passive diffusion. This is consistent with the glucose-mediated effects on the tight junction area and membrane fluidity.

In conclusion, high extracellular glucose concentration in isotonic media significantly alters physical barrier properties of Caco-2 cell monolayers that predominantly restrict transepithelial transport of solutes permeating the cell barrier by paracellular and transcellular passive diffusion and facilitated transport mediated by PepT-1.

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